

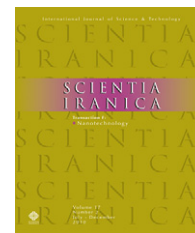


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Spectrophotometric determination of glutathione and cysteine based on aggregation of colloidal gold nanoparticles

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KEYWORDS

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Abstract We report herein the development of a highly sensitive colorimetric method for the determination of cysteine and glutathione, based on aggregation of the citrate capped gold nanoparticles (Au NPs). This was exploited from high affinity of low-molecular-weight amino thiols towards the Au NPs surface, which could induce displacement of the citrate shell by the thiolate shell of target molecules, resulting in aggregation of the NPs through intermolecular electrostatic interaction or hydrogen-bonding. As a result of aggregation, which can be affected by the ionic strength, pH and concentration of Au NPs, the plasmon band at around 521 nm decreases gradually, along with formation of a new red shifted band. The calibration curves, which are derived from the intensity ratios of absorbance at 640 nm and 650 nm for cysteine and glutathione, respectively, to the original wavelength of 521 nm, display a linear relation in the range of 1×10^{-6} – 100×10^{-6} M cysteine and 5×10^{-6} – 200×10^{-6} M glutathione. The obtained detection limits (3σ) were 2.1×10^{-6} M and 3.3×10^{-6} M for determination of cysteine and glutathione, respectively.

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1. Introduction

The determination of low-molecular-weight amino thiol based biological molecules, such as cysteine (Cys) and glutathione (GSH) (Scheme 1), in human plasma are becoming increasingly important, due to exploration of their crucial role in many biological pathways. Cysteine is sulfur containing non-essential amino acids, which is critical to the metabolism of a number of essential biochemicals, including coenzyme A, heparin, biotin, and glutathione. The tripeptide glutathione (GSH), a master antioxidant which is supplied by the cysteine, is the main intracellular defence against oxidative stress, in addition to regulating the action of antioxidants, such as vitamins C and E, in the body. Recent studies have shown the significant

contribution of cysteine and glutathione in HIV, diabetes, liver damage, heart diseases, mild cognitive impairment, edema, pneumonia, skin lesions and other serious diseases [1–11]. Therefore, it is very important to find a sensitive, accurate and simple method for the measurement of cysteine and glutathione.

The majority of the currently available methods relating to the detection of cysteine and glutathione are based on electrochemical, immunoassay and chromatography techniques that benefit from derivatization with chromophores or fluorophores, or often carried out in conjunction with HPLC, GC, MS and capillary electrophoresis [12–23]. Although these methods can well sense amino thiols, their practical applications are limited because of suffering from some inherent drawbacks, such as requiring complicated instrumentation, expensive biological reagents or cumbersome sample preparation.

Gold nanoparticles (Au NPs) have received great attention for potential biological analysis, in recent years, due to the Surface Plasmon (SP) resonance phenomena, which is responsible for their unique size and shape optical dependant properties [24,25]. Having high order of magnitude for extinction coefficients, in addition to the dependence of surface plasmon resonance wavelength and intensity on the dielectric constant

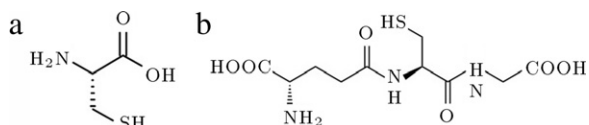
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Scheme 1: Schematic illustration of the structures of (a) cysteine, and (b) glutathione.

of the medium, Au NPs are emerging as a promising tool for analysis of biologically active molecules through colorimetric assay [26–28].

Herein, we have developed a simple and highly sensitive colorimetric method for detection of both cysteine and glutathione based on the aggregation size-dependent optical property of citrate capped spherical Au NPs, through employing the strong affinity characteristics of the thiol group towards the surface of the Au NPs. The effects of affecting parameters, including concentration of NPs, ion strength and pH, have been investigated to find the optimized condition, with highest sensitivity and selectivity.

2. Reagent and apparatus

Thiol-containing amino acids, cystine (Cys, 97%) and glutathione (Glu, 98%), and all other chemicals, such as hydrogen tetra-chloroaurate (HAuCl₄, 99%), sodium citrate (99%), were purchased from Merck and were used without further purification. For the preparation of all samples, water purified with cartridges from Millipore (Milli-Q) to a resistivity of 18 MΩ was used. UV–vis spectroscopy was performed on a Lambda 25 (Perkin-Elmer, USA) spectrophotometer with the use of a 1.0 cm glass cell. Measurements of pH were made with a Denver Instrument Model of 270 pH meter equipped with a Metrohm glass electrode. Size distributions of the particles were obtained using Zetasizer Viscotec 802 at ambient temperature. Transmission Electron Microscopy (TEM) images were recorded with a PHILIPS MC 10 TH microscope at an acceleration voltage of 100 KV.

3. Experimental

3.1. Procedure for preparation of Au NPs

Citrate-capped Au NPs with an average diameter of about 13.1 ± 1.3 nm were synthesized following the method pioneered by Turkevich et al. [29,30] in which a 50 mL solution containing 1 mM of HAuCl₄ was prepared and heated under reflux. At the boiling point, 5 mL of 38.8 mM trisodium citrate were added to this solution under vigorous stirring and the mixture was heated under reflux for an additional 30 min during which the color changed to deep red, indicating the formation of Au NPs. The solution was set aside to cool to room temperature and stored at 4 °C for further utilization. The particle concentration at the resulted solutions was estimated to be 15 nM, according to Beer's law, and the extinction coefficient (ϵ) of about 13 nm Au NPs at 520 nm [31].

3.2. Procedure for determination of cysteine and glutathione

For the determination of cysteine, the solutions containing 2 mL of as-prepared Au NPs and 8 mL of Milli-Q water were prepared in volumetric flasks. To these solutions, 100 μ L of 1.0 M NaCl and different concentrations of cysteine were added, one by one in order. For the determination of glutathione, the

same procedure was performed on a mixture containing 1 mL of synthesized Au NPs and 9 mL of Milli-Q water, with pH adjusted at 4.7 by 0.1 M of HCl. The absorbance spectra were recorded at 3 min intervals, shortly after adding the analytes. It should be noted that the order of the addition of the reagents is very important. Alteration of the order of the addition of reagents causes inaccurate results, with a low level of reproducibility.

4. Results and discussions

Thiol-containing amino acid molecules have high affinity towards the surface of gold nanoparticles that can be finely described by the hard–soft acid–base theory [32]. Displacement of the citrate group shell with thiol-containing amino acids, such as cysteine and glutathione, induces the aggregation process, due to the hydrogen binding or electrostatic interaction between non-covalently adsorbed thiol-containing amino acids. As a result of the aggregation, the surface plasmon resonance absorption of Au NPs around 521 nm decreases, along with formation of a new band at longer wavelength. This is due to the near-field coupling in the resonant wavelength peak of the interacting particles [33]. Figure 1 displays typical TEM and DLS images and corresponding UV–vis absorption spectra of Au NPs, before and upon the addition of certain quantities of cysteine in appropriate ion strength. This clearly shows the effect of aggregation on surface plasmon maxima, size and optical properties of the Au NPs.

To establish the optimum analytical conditions for detection of cysteine and glutathione, the effects of critical parameters, including ion strength, pH and concentration of Au NPs, have been investigated.

4.1. Effect of ion strength

Ion strength has a crucial role in the aggregation process that can be attributed to the ability of strong electrolytes to constrict the aroused electrical double-layer from the capping agent. We observed that in the absence of strong electrolytes (low ion strength), Au NPs did not undergo the aggregation, even at the high concentration of cysteine or glutathione (Figure 2). Also, it is found that by increasing the ion strength above a certain limit, the aggregation of nanoparticles induces even in the absence of analytes. Therefore, some controlled experiments were conducted that have revealed the concentration of 10 mM of NaCl as optimized in which there is aggregation not in the absence, but only in the presence of our target analytes.

4.2. Effect of pH

Because of the presence of hydroxyl, carboxyl and amine groups in cysteine and glutathione, pH is another critical parameter that should be taken into consideration. As shown in Scheme 2 (a), electrostatic interactions are the mainly responsible for aggregation of Au NPs in the presence of cysteine. With this in mind, to increase the possibility of electrostatic interaction, the best condition is attainable in which the cysteine molecule is available in the form of zwitterions. This also could be confirmed based on the stock diagram of cysteine in which the zwitterion form has a greater contribution in the range of pH = 3–pH = 7 [34]. As shown in Figure 3, the synthesized Au NPs are stable in the range of pH of 4.5 and 10. Therefore, further adjustment of pH was eliminated in the case of cysteine. Based on the fact that the initial pH of synthesized Au NPs is found to be about 6.

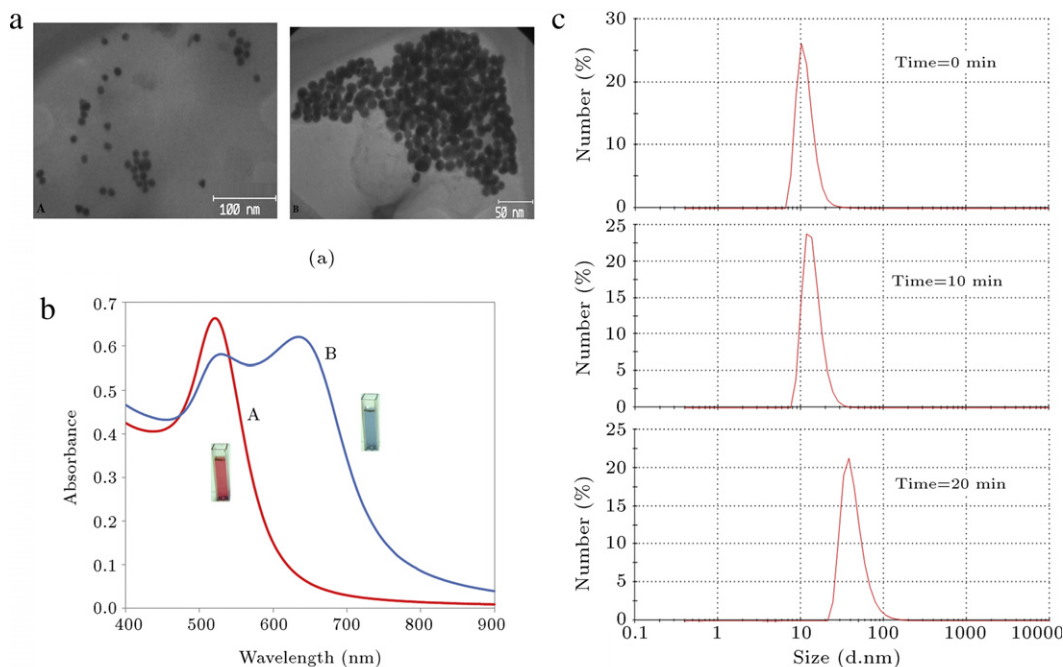


Figure 1: (a) Typical TEM images; (b) corresponding UV-vis absorption spectra; and (c) size distribution of the Au NPs before and upon the addition of certain quantity of cysteine.

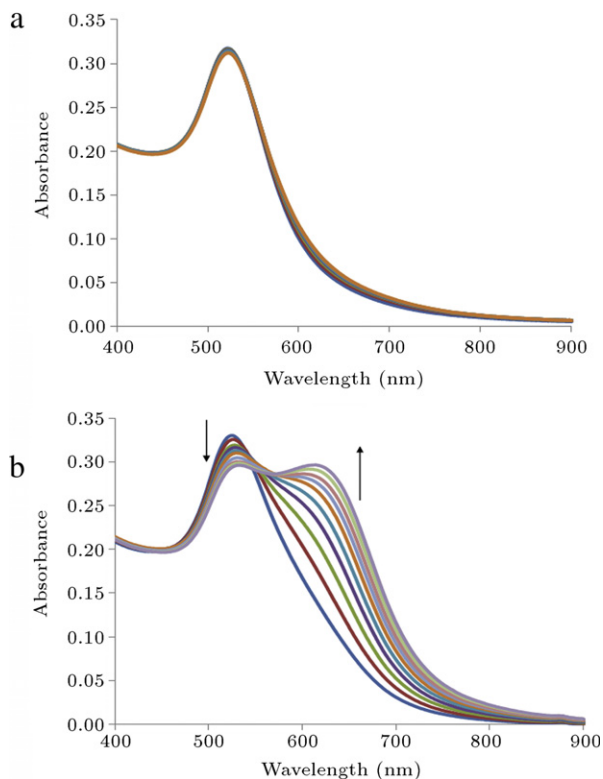
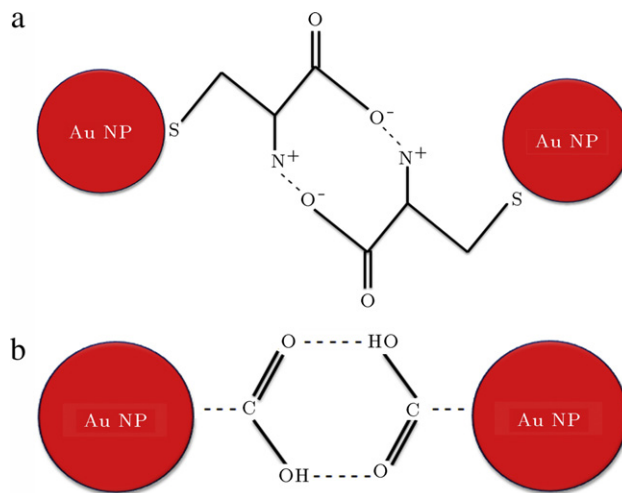


Figure 2: Variation in the surface plasmon maxima of Au NPs in the presence of 10 μ M cysteine (a) in the absence of NaCl, and (b) upon the addition of 100 μ L of 10 mM NaCl (time interval to record each spectrum is 3 min).

The aggregation mechanism for glutathione (Scheme 2(b)) benefits from a hydrogen bonding strategy, which demands a very precise view over the controlling of pH. We found,



Scheme 2: Schematic illustration of the aggregation mechanism for (a) cysteine, and (b) glutathione.

in the case of glutathione, no aggregation could be induced at the initial pH of the synthesized Au NPs (pH 6). This can be ascribed to the electrostatic repulsion between negatively charged carboxylic groups in the forms of zwitterions ($\text{NH}_3^+/\text{COO}^-/\text{SH}/\text{COO}^-$) and negatively charged citrate shells. However, by adjusting pH at acidic environment at which the carboxylic groups have their protonated form, the aggregation could be induced. We also investigated the anti-aggregation process for glutathione in which the color and corresponding UV-vis absorption spectra of the aggregated solution of glutathione-Au NPs were restored to the initial state, upon increasing the pH at 40 $^\circ\text{C}$ (Figure 4), due to the weaker hydrogen bonding interactions. This further confirmed contribution of hydrogen bonding regarding aggregation of Au NPs in the presence of

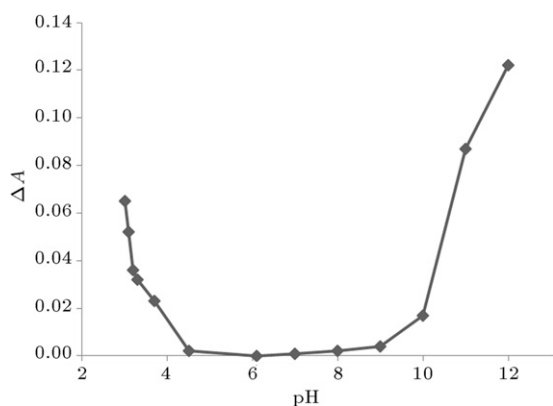


Figure 3: Effect of pH on the self-aggregation of Au NPs in the absence of analytes.

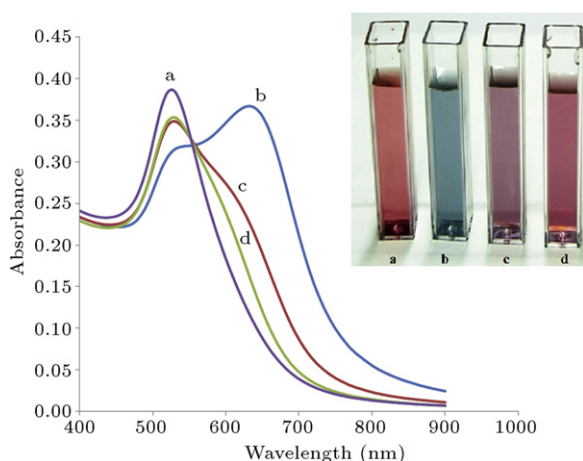


Figure 4: Anti-aggregation of the glutathione-induced aggregated Au NPs by increasing the pH at 40 °C. (a) Au NPs at pH = 6; (b) aggregated particles at pH = 6.0; (c) at pH = 7.0; and (d) at pH = 9.0.

glutathione. Hence, by considering the fact that Au NPs are not stable at a pH below 4.5, we proposed pH 4.7 for further experiments relating to the determination of glutathione.

4.3. Effect of concentration of Au NPs

To further optimize the sensitivity and selectivity of the method, we investigated the effect of the concentration of Au NPs on the aggregation process in the presence of 10 μM cysteine and 10 mM NaCl. As shown in Figure 5(a), for cysteine, the rate of aggregation increases with an increase in the concentration of NPs. However, higher concentrations of gold nanoparticles suffer from a limited linear range of detection. Therefore, based on the preliminary experiments, a concentration of 3 nM of the NPs at the final solution was selected for further experiments, which benefits from both acceptable linear range and reasonable time to complete the detection process. However, the obtained results for glutathione have revealed a decrease in the rate of aggregation by increasing the concentration of nanoparticles as shown in Figure 5(b). Herein, the concentration of 1.5 nM was selected for further measurements.

4.4. Analytical figures of merit

The linear ranges for determination of cysteine and glutathione were evaluated under their optimum conditions.

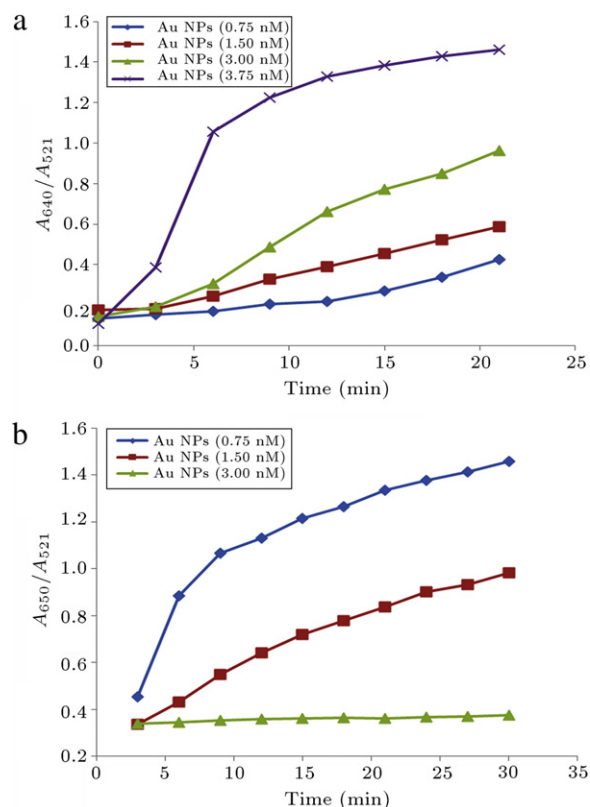


Figure 5: Effect of NPs concentration on aggregation rate in the presence of (a) 10 μM of cysteine, and (b) 20 μM of glutathione.

Figure 6(a) shows a linear relation between the ratio of A_{640}/A_{521} and concentration of cysteine in the range of 1.0–100 μM . For glutathione, a linear range could be achieved between the ratio of A_{650}/A_{521} and concentration of 5–200 μM (Figure 6(b)). The lower limit of detection for determination of cysteine and glutathione at a signal-to-noise ratio of 3 (3σ) were 2.1 μM and 3.3 μM , respectively. The study of precision, which was made with five independent experiments, revealed relative standard deviations (% RSD) of 1.8% and 4.1% for determination of 10 and 20 μM of cysteine and glutathione, respectively.

4.5. Interference study

To assess the selectivity of the proposed method, an analysis of a standard solution of cysteine (10 μM) and glutathione (20 μM) were independently conducted, under their optimum conditions and in the presence of foreign metal ions and amino acids, with the possibility of interference. As shown in Figure 7, a wide range of presented species do not interfere, even at concentrations 50 times higher than those of the analytes.

4.6. Real sample analysis

The optimized method was applied for the determination of cysteine and glutathione in Ringer's serum as a real sample. Certain amounts of cysteine and glutathione were independently spiked into the real samples, and subsequently their concentrations were determined in each sample. The results given in Table 1 show the potential and feasibility of the developed method for determination of cysteine and glutathione in the real samples. It should be noted that

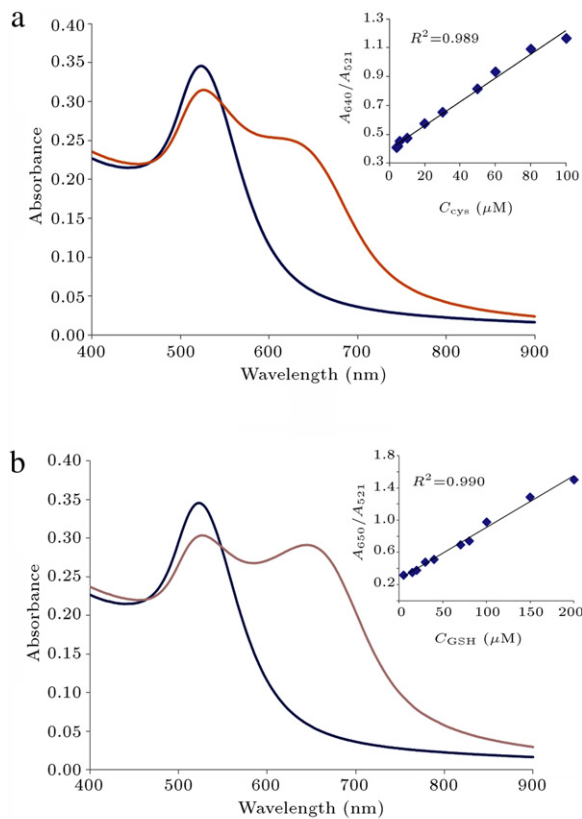


Figure 6: A typical absorption spectra of Au NPs upon adding certain quantity of (a) cysteine, and (b) glutathione (the insets are corresponding calibration curve in the ranges of 1–100 μM and 5–200 μM for cysteine and glutathione, respectively).

Table 1: Determination of cysteine and glutathione in Ringer's serum.			
Analyte	Spiked (μM)	Measured (μM)	Recovery %
Cysteine	30	32.35	108
	50	52.29	104
	70	73.10	104
	15	16.2	108
Glutathione	50	51.25	102
	170	174.13	102

Table 2: Determination of cysteine in the presence of glutathione.			
Glutathione (μM)	Spiked cysteine (μM)	Measured cysteine (μM)	Recovery (cysteine) %
200	10	9.6	96
200	15	15.68	104
200	20	20.36	102
200	30	29.4	98

glutathione is usually considered the most predominant interference for determination of cysteine. Therefore, some experiments were conducted in order to determine cysteine in the presence of glutathione. For this purpose, different quantities of cysteine were spiked into the solution of the ringer serum containing 200 μM of glutathione. It is found that by controlling pH at 6.0 in which the glutathione molecules could not affect the aggregation process, the cysteine quantity could be measured with an acceptable recovery percentage as shown in Table 2.

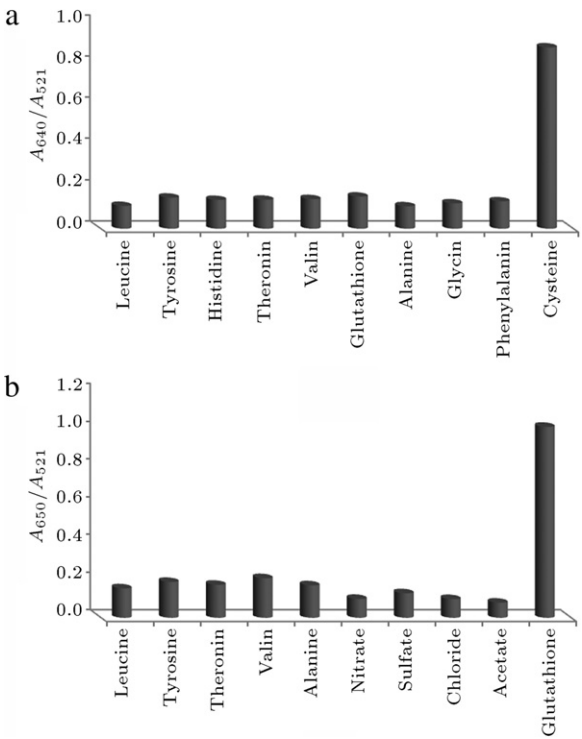


Figure 7: The interference study for (a) 10 μM of cysteine (at pH 6.0), and (b) 20 μM of glutathione (at pH 4.7) in the presence of interfering species with concentration of 50 times higher than those of analytes.

5. Conclusion

In summary, the potential and feasibility of Au NPs for the colorimetric determination of cysteine and glutathione have been demonstrated. The developed methodology, based on analyte-induced aggregation of NPs, could achieve a quantification limit at low levels, and good linearity, accompanied by acceptable accuracy and reproducibility, permitting determination of cysteine and glutathione in the ringer serum. There are further developments to investigate its clinical potential for determination of trace amounts of other targets of interest in biological samples.

Acknowledgments

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